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# Morpho-functional effects of heat stress on the gills of Antarctic *T. bernacchii* and *C. hamatus*

Filippo Garofalo[a], Gianfranco Santovito[b], Daniela Amelio[a,*]

[a] Department of Biology, Ecology and Earth Sciences (B.E.S.T.), University of Calabria, Arcavacata di Rende, CS, Italy
[b] Department of Biology, University of Padova, Padova, Italy

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ABSTRACT

The effect of increasing ocean water temperature on morpho-functional traits of Antarctic marine species is under intense attention.

In this work, we evaluated the effects of acute heat stress on the gills of the Antarctic haemoglobinless *Chionodraco hamatus* and the red blooded *Trematomus bernacchii* in terms of morphology, heat shock response, antioxidant defense and NOS/NO system. We showed in both species that the exposure to high temperature (4 °C) induced structural alterations, such as epithelial lifting and oedema of secondary lamellae. By immunolocalization we also observed that HSP-90, HSP-70, Xantine Oxidase, Heme Oxigenase and NOS are expressed in both species under control conditions. After heat stress the signals increase in *C. hamatus* being absent/or reduced in *T. bernacchii*. Our preliminary results suggest a specie-specific morpho-functional response of the gills of the two Antarctic teleosts to heat stress.

## 1. Introduction

Antarctic fish are extreme stenotherm animals, adapted to live at temperatures close to the freezing point of ocean water (−1.9 °C). They experience stable annual temperature fluctuations less than 1 °C (Hunt et al., 2003). Due to climate changes and the consequent increment in water temperature, Antarctic species are exposed to stressful conditions. Data in Antarctic research stations showed in Antarctic Peninsula that average temperatures increased more than 3 °C between 1949 and 1996 in the atmosphere (King and Harangozo, 1998), and over the last 60 years, of 1 °C in the contiguous ocean surface waters (Qiu, 2012).

Some Antarctic fish, including *T. bernacchii*, showed higher thermal plasticity after acclimation periods (Podrabsky and Somero, 2006; Robinson and Davison, 2008; Bilyk and DeVries, 2011; Strobel et al., 2012, 2013). However other species are less tolerant to temperature increment (4 °C) (Robinson, 2008), and this has been associated to heat-induced hypoxia (Pörtner et al., 2004; Pörtner, 2010).

Exposure to temperatures, different from those to which the fish is adapted, can promote important morpho-functional changes at cell, tissue and organ level (Hwang et al., 2011). The gills, being directly exposed to environment, are extremely susceptible to chemical and physical factors, including thermal changes (Hwang et al., 2011). In the Antarctic teleosts, the response of the gills is only partially known and limited conflicting data are available. In the gills of *Notothenia rossii* heat stress is associated to hypoxia and oxidative damage (Forgati et al., 2017). However, in Antarctic teleosts subjected to heat-induced hypoxia, an increase in enzymatic antioxidant levels has been reported (Forgati et al., 2017). Tissue hypoxia is also associated with activation of the Nitric Oxide Synthase (NOS), with consequent formation of reactive nitrogen species (RNS) including nitric oxide (NO) (Hochachka and Lutz, 2001; Rahman and Thomas, 2018). In Antarctic teleosts the presence of this enzymatic system has been documented, by immunolocalization and biochemical approches. In particular, in different Antarctic species such as *T. bernacchii*, *C. hamatus* and *Chaenocephalus aceratus*, endothelial and inducible NOS (eNOS and iNOS respectively) are present (Amelio et al., 2006; Garofalo et al., 2009a; Garofalo et al., 2009b). In the heart of Antarctic species, as the red blooded *Notothenia coriiceps* and hemoglobinless *C. aceratus*, the hypoxia inducible factor (HIF1-α) has been sequenced, although the long evolution of the Notothenioidei in the oxygen-rich waters may have reduced selective pressure to maintain a hypoxic response (Rix et al., 2017). The molecule is highly conserved with respect to other teleosts, even if contains an insert of variable length between Antarctic species, which may affect dimerization of HIF-1α and translocation into the nucleus and/or DNA binding (Rix et al., 2017).

As shown in many vertebrates heat stress is also implicated in the generation of reactive oxygen species (ROS), due to heat-induced increase of metabolic rate (Hochachka and Somero, 2002; Bagnyukova

* Corresponding author.
*E-mail address:* daniela.amelio@unical.it (D. Amelio).

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et al., 2003) and damaged heme groups (Girotti, 1998). Oxidative stress causes oxidative damage to macromolecules, including, enzyme inactivation, lipid peroxidation (LPO), and DNA damage, that compromise organ functions and may lead to cell death (Halliwell and Gutteridge, 2007).

Exposure to stresses, including high temperature and oxidant injury, induces an over-expression of Heat Shock Proteins (HSPs), enabling organisms to resist the insults of adverse stressors so as to maintain cell homeostasis and survival (Ming et al., 2010). These proteins are also present under normal conditions, playing a role as molecular chaperones that protect proteins from denaturation, being involved in folding of nascent protein and in removing irreversibly damaged proteins (Pelham, 1986; Geething and Sambrook, 1992).

Based on these premises, the objective of this study was to evaluate, in the Antarctic hemoglobinless *C. hamatus* and red blooded *T. bernacchii*, the effects of thermal stress, 4 °C for 10 days, on the branchial morphology, heat shock response, antioxidant defense and NOS/NO systems.

Since thermal tolerance of Antarctic Notothenioid correlates with level of circulating hemoglobin, we choice to compare the hemoglobin natural knockout *C. hamatus*, with the red blooded *T. bernacchii*. In particular species with lower/absent values of hemoglobin showed minor thermal tolerance. Therefore icefishes are particularly sensitive to temperature elevation because of a lack of hemoglobin and may be considered a sentinel taxon for temperature change (Beers and Sidell, 2011).

Our study revealed the presence of morphological changes in the gills of the two Antarctic teleosts species after heat stress. We also found that HSP-90, HSP-70, Xantine Oxidase, Heme Oxigenase and NOS are expressed in both species under control conditions. Following the exposure to temperature increase, their expression is enhanced in *C. hamatus* whereas is reduced or disappeared in *T. bernacchii*. On the whole, our results suggest a specie-specific morpho-functional response of the two Antarctic teleosts to heat stress.

## 2. Material and methods

### 2.1. Animals

The study was conducted on 12 specimens of both sexes, of icefish *C. hamatus* (N = 6) and red-blooded *T. bernacchii* (N = 6), weighing 333 ± 17 (mean ± S.E.M.). Fish were collected in the proximity of Mario Zucchelli Station in Terra Nova Bay, Antarctica (74°42′S, 167°7′E) and kept in aquaria supplied with aerated seawater at approximately 0 °C. After capture, animals were divided in two groups: control (CTRL; n = 3 for each species) and heat stress exposed (HS10; N = 3 for each species). The CTRL was maintained for at least 10 days in aerated, running seawater at temperatures of 0 °C, whereas HS10 was exposed at heat stress (4 °C) for 10 days. Before sacrifice, fishes were anesthetized with 0,2 g $l^{-1}$ MS222 (tricaine methane sulfonate, Sigma-Aldrich Chemical Co., UK). In accordance with the accepted standards of animal care, experiments were organized to minimize stress and number of animal used. The sample collection and animal research conducted in this study comply with Italy's Ministry of Education, University and Research regulations concerning activities and environmental protection in Antarctica and with the Protocol on Environmental Protection to the Antarctic Treaty, Annex II, Art. 3. All experiments have been performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

### 2.2. Morphological analysis

Gill samples of *T. bernacchii* and *C. hamatus* of both CTRL and HS10 were flushed in phosphate-buffered saline (PBS), fixed in a MAW (methanol:acetone:water = 2:2:1) solution, dehydrated in graded ethanol (90% and 100%), cleared in xylol, embedded in paraplast (Sigma-Aldrich Chemical Co., UK), and serially sectioned at 8 µm. Sections were placed onto Superfrost Plus slides (Menzel-Glaser, Braunschwerg, Germany), deparaffined in xylene, and rehydrated in an alcohol gradient. Several sections were stained with hematoxylin-eosin for a general assessment of tissue structure.

### 2.3. Immunoenzimatic detection

Rehydrated gill sections were processed by immunohistochemical staining using a HRP/DAB detection kit (Abcam, Cambridge, MA, USA). Briefly, sections were deparaffined and rehydrated in TBS and pretreated with $H_2O_2$ to remove endogenous peroxidase activity. They were incubated for 1 h with Protein Block, and overnight (4 °C) with rabbit polyclonal anti ARC (apoptosis repressor with caspase recruitment domain), HIF-1α (Hypoxia Inducible Factor), HSP-90 and mouse anti HSP-70 (Heat Shock Protein) antibodies (1:100; Santa Cruz Biotechnology, Inc., Heidelberg, Germany). Slides were washed in TBS, incubated with Biotinylated goat anti-rabbit/or anti-mouse IgG and then with streptavidine peroxidase complex. The signal was visualized by using diaminobenzidine (DAB) as chromogen.

### 2.4. Immunofluorescence

Parallel rehydrated sections were rinsed in TBS and incubated with 1.5% BSA in TBS for 1 h. They were then incubated overnight at 4 °C with mouse monoclonal antibodies directed against Xantine Oxidase (XO), Haeme Oxigenase (HO), neuronal nitric oxide sinthase (nNOS) (Santa Cruz Biotechnology, Inc., Heidelberg, German) and rabbit polyclonal antibody directed against endothelial NOS (eNOS) (Sigma-Aldrich Chemical Co., UK) diluted 1:100 in TBS. In teleosts, a canonical eNOS seems to be absent (Andreakis et al., 2011). Nevertheless, by different methodological approaches including immunolocalization with heterologous mammalian antibodies has been showed the presence of an "eNOS-like" activity in the different organs of several teleost species (Amelio et al., 2008; Imbrogno et al., 2013; Gary et al., 2015). For signal detection, after washing in TBS, slides were incubated with FITC-conjugated anti-mouse or anti-rabbit IgG (Sigma-Aldrich Chemical Co., UK, 1:100) and mounted with mounting medium (Vectashield, Vector Laboratories Burlingame, CA, USA). Negative controls were obtained on parallel sections treated in the same manner, excluding the primary antibody.

### 2.5. Apoptosis detection

TUNEL staining was performed with *in Situ* Cell Death Detection Kit (POD from Roche Diagnostics, Germany), according to the manufacturer. Briefly, rehydrated sections were incubated with proteinase K (20 µg/mL) at 37 °C for 20 min. Subsequently, slides were washed twice with PBS, and endogenous peroxidase was quenched with 0.3% $H_2O_2$ in PBS for 15 min. They were then rinsed and incubated with TUNEL at 37 °C in a humidified box for 60 min, mounted with mounting medium (Vectashield, Vector Laboratories Burlingame, CA, USA), and observed under fluorescence microscope (Axioscope, Zeiss, Oberkochen, Germany). Negative controls were performed by using the same protocol without terminal deoxynucleotidyl transferase (TdT) enzyme.

## 3. Results

### 3.1. Morphological evaluations

Under control (0 °C) conditions, the gills of *C. hamatus* (Fig. 1A) and *T. bernacchii* (Fig. 1C), showed the typical morphology of teleost gills (Maina, 2002) characterized by primary and secondary lamellae enveloped by respiratory epithelium and divided into vascular channels by polygonal pillar cells. In the presence of thermal stress (4 °C), in both

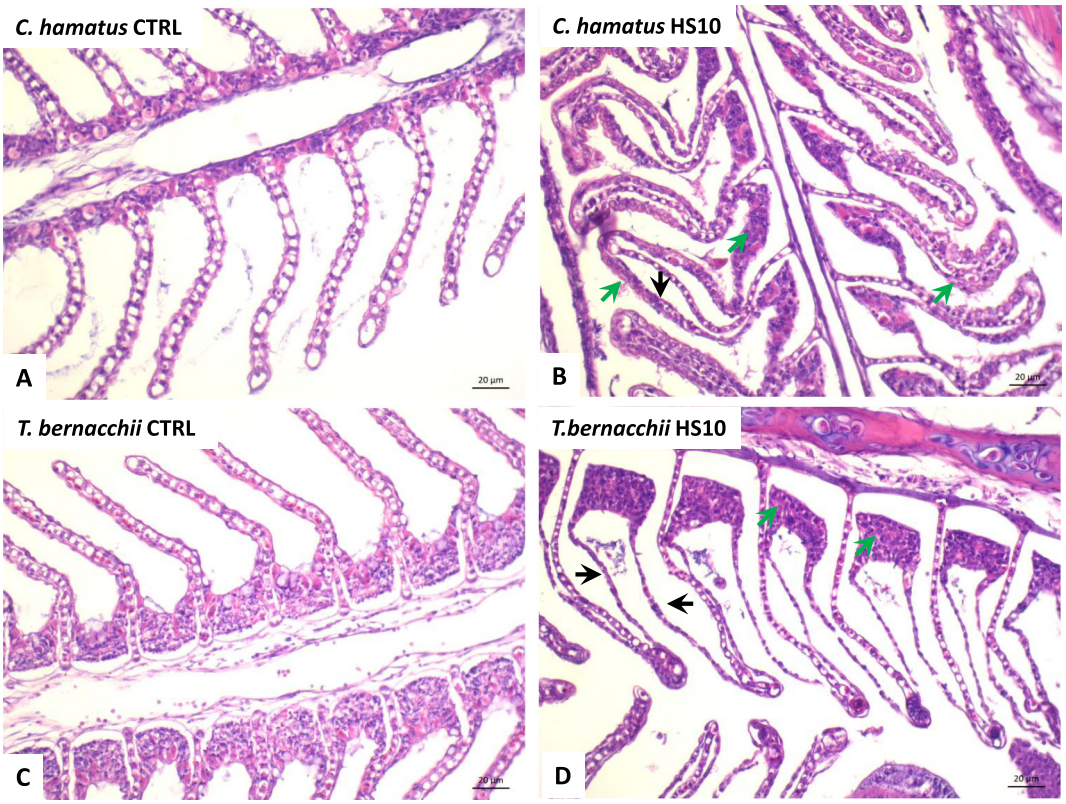


**Fig. 1.** Hematoxylin–eosin stained sections illustrating basic histological features of the *C. hamatus* (A,B) and *T. bernacchii* (C,D) gills under control (CTRL A,C) and heat stress (HS10 B,D) conditions. Epithelial lifting (black arrows); Hyperplasia (green arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

*C. hamatus* (Fig. 1B) and *T. bernacchii* (Fig. 1D) acclimated at high temperature the branchial morphology was characterized by structural alterations, mainly represented by epithelial lifting and oedema of secondary lamellae, proliferation and hypertrophy of mucocytes, and epithelial hyperplasia. In particular epithelial hyperplasia of secondary lamellae is more evident in *C. hamatus* (Fig. 1B) than in *T. bernacchii* (Fig. 1D).

### 3.2. Physiological responses

The presence of HSP-90, HSP-70, HIF-1α, and ARC was evaluated by immunoenzimatic methods, both in the icefish *C. hamatus* and in the red blooded *T. bernacchii* under CTRL and HS10 conditions. HSP-90 was detected in the branchial epithelium of both CTRL (Fig. 2A) and HS10 (Fig. 2B) *C. hamatus*. On the contrary, in *T. bernacchii*, HSP-90 was absent under both conditions (Fig. 2C,D). Incubation with anti HSP-70 antibody revealed under control conditions the presence of the protein in both teleost species. In *C. hamatus* HSP-70 mainly localized at the level of mucocytes located at the base, and in the epithelium of secondary lamellae (Fig. 2E). With respect to CTRL conditions (Fig. 2E) in HS10 *C. hamatus* (2F) the signal appeared stronger, while it is absent in *T. bernacchii* (data not shown).

Immunolocalization of HIF-1α on parallel sections revealed that in *C. hamatus* (CTRL), the protein was prevalently expressed in the mucocytes at the base of lamellae (Fig. 3A). In icefish exposed to thermal stress, the signal was stronger than in CTRL animals and localized also in the mucocytes interspersed in the respiratory epithelium (Fig. 3B). In *T. bernacchii* (Fig. 3C,D) the signal is weaker with respect to *C. hamatus*. The exposure to high temperature was accompanied by a relatively low increment of HIF-1α expression, which appears confined only at the endothelial level (Fig. 3D).

The presence of the enzymes eNOS, nNOS, XO and HO was also detected by immunofluorescence in the gills of *C. hamatus* and *T. bernacchii*. In the icefish, both eNOS (Fig. 4A,B) and nNOS (Fig. 4C,D) localized at the epithelial level of the secondary lamellae of CTRL (4 A,C) and HS10 (4 B,D) animals. In *T. bernacchii* (CTRL) a very low eNOS (Fig. 4E) and nNOS (Fig. 4G) signal was detected only at the level of vascular endothelium, whereas both enzymes were not present in HS10 samples (Fig. 4F,H).

In *C. hamatus* of both CTRL and in HS10 (Fig. 5A,B) groups XO localized in the small vessels of secondary lamellae. The signal appears stronger in the pillar cells of the HS10 (Fig. 5D) specimens respect to CTRL (Fig. 5C). On the contrary, XO expression was absent in *T. bernacchii* of the two experimental groups (Fig. 5E,F).

In CTRL *C. hamatus* HO enzyme localized in the epithelium which envelope both primary and secondary lamellae at level of mucocytes (Fig. 6A,C). In the HS10 group the signal was stronger and homogenously distributed (Fig. 6B,D) with respect to CTRL. No HO signal was detected in the gills of *T. bernacchii* (Fig. 6E,F).

### 3.3. Apoptosis

Under CTRL conditions, the gills of both Antarctic teleosts showed the presence of apoptosis identified by fluorescent nuclei (Fig. 7A,C). After the exposure to high temperature, these nuclei notably increase in *T. bernacchiii* (Fig. 7D) while they were strongly reduced in *C. hamatus* (Fig. 7B). To support these data an immunoenzimatic method was used to describe the localization of ARC. Results showed the presence of ARC in both species under CTRL conditions (Fig. 8A,C). The signal appears mainly confined at the level of some pillar cells located in the proximal extremity of the secondary lamellae. In the HS10 conditions, the signal was strongly augmented in the pillar cells of the gills of *C. hamatus* (Fig. 8B), whereas in *T. bernacchii* it was not detected (Fig. 8D).

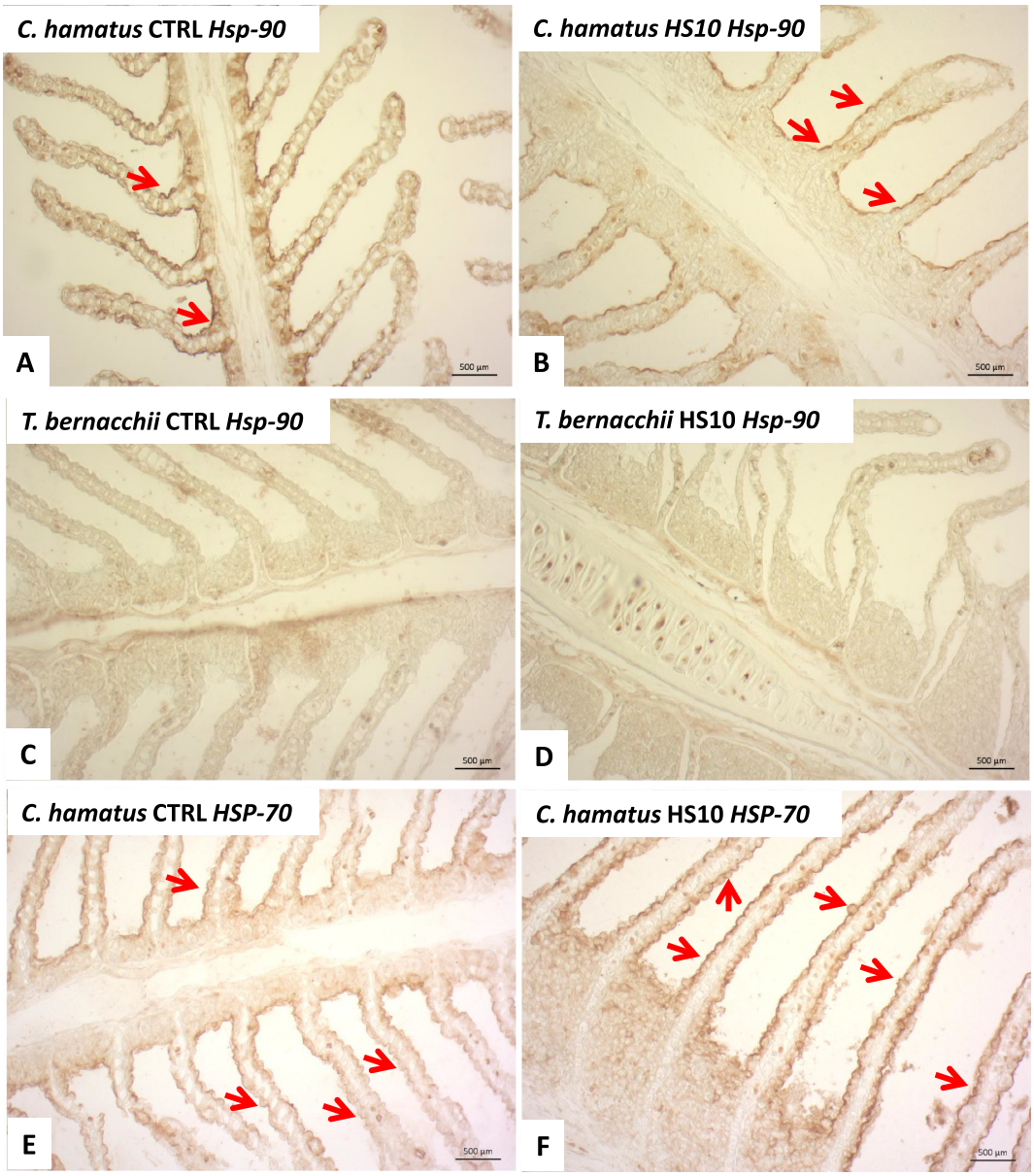


**Fig. 2.** Immunohistochemical localization of the heat shock protein 90 (HSP-90; A-D) and HSP-70 (E,F) in the gills of *C. hamatus* (A,B,E,F) and *T. bernacchii* (C,D). In both CTRL (A,E) and HS10 (B,F) gills of *C. hamatus* the signal was confined at the epithelial level (red arrows). In *T. bernacchii* the signal is absent. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 4. Discussion

Antarctic teleost are extremely sensitive to water thermal regimes. As a consequence, they can be importantly affected by the progressive increase of ocean temperature due to global warming.

In this work, we evaluated, on the gills of the Antarctic haemoglobinless *C. hamatus* and its red-blooded counterpart *T. bernacchii*, the effects of acute heat stress on the morphology, the heat shock response, the NOS/NO system and the antioxidant defense.

The gills, due to their close contact with water, are directly affected by environmental modifications, including temperature changes (Hwang et al., 2011; Truzzi et al., 2018a). They are significantly involved in the communication between water and blood thanks to their extremely thin diffusive barrier (Wood, 2001). Therefore, morphological and/or molecular changes occurring on the gills can interfere with crucial functions such as respiration, ion-regulation and nitrogenous waste excretion.

In the present study we showed that in both *C. hamatus* and *T. bernacchii*, heat stress was associated with an important structural branchial alteration exemplified by epithelial lifting and hyperplasia, oedema of secondary lamellae, proliferation and hypertrophy of mucocytes. Similar morphological alterations were observed in previous studies on Antarctic fish gills exposed to different pollutants (Flores-Lopes and Thomaz, 2011) suggesting that the gills respond to stressful conditions undergoing a remarkable morphological rearrangment. In our study, epithelial hyperplasia and lifting were observed in both Antarctic teleost exposed to thermal stress. However, in *C. hamatus* hyperplasia was more evident than in *T. bernacchii*. This structural change is of interest since suggests, particularly in the icefish, an increase of the diffusional distance between water and blood with a possible consequent reduction of the efficiency in branchial $O_2$ supply and $CO_2$ removal.

It is known that in fish heat stress can induce hypoxia (Pörtner et al., 2004, Pörtner, 2010). With respect to other organs, the gills are much more affected by heat stress-dependent hypoxia. In fact, high temperatures are associated with a reduction of the concentration of water

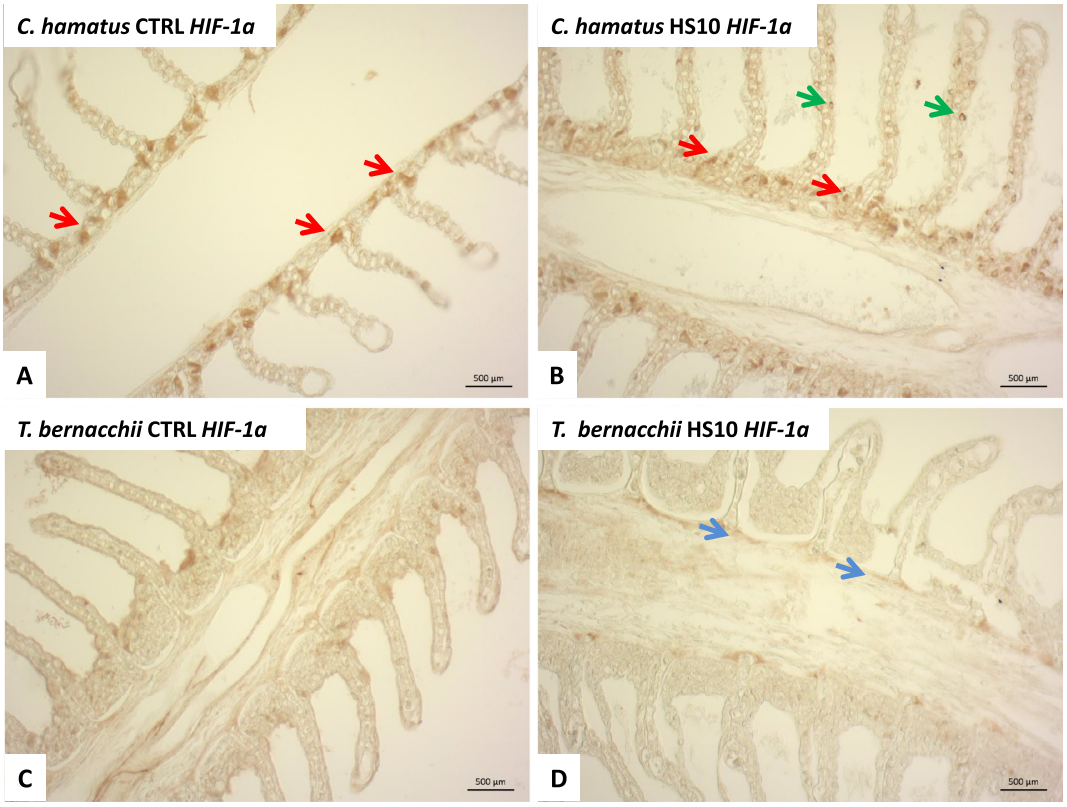


**Fig. 3.** Immunohistochemical localization of the hypoxia inducible factor (HIF-1α) in the gills of *C. hamatus* (A,B) and *T. bernacchii* (C,D). In CTRL (A) *C. hamatus* the signal was mainly expressed in the mucocytes at the base of lamellae (red arrows). After heat stress (B), the signal is stronger and confined also in the mucocytes interspersed in the respiratory epithelium (green arrows). In *T. bernacchii* a weak signal was detected only after HS10 and is confined at the endothelial level (blue arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

dissolved $O_2$, and an increase of $O_2$ absorption rate due to the high branchial metabolic rate (Das et al., 2004). Therefore gills are the first tissue affected by $O_2$ depletion (Evans et al., 2005). Accordingly, we evaluated whether in *C. hamatus* and *T. bernacchii* the gills undergo hypoxia after exposure to thermal increase. For this purpose, we analyzed, by immunolocalization, the branchial expression of HIF-1α, a major marker of hypoxia, that recently was found to be influenced by heat stress, at least in mammals (Ely et al., 2014). Contrarily to mammalian cells in which HIF-1α is detectable only under severe $O_2$ deprivation, we found that this protein was expressed in the gills of both Antarctic species exposed to normal temperature. This is in line with previous data showing that, in fish tissues, HIF-1α is expressed also under unstressed conditions (Stroka et al., 2001; Rissanen et al., 2006; Heise et al., 2006a, 2006b). We also found that HIF-1α expression increases in the gills of *C. hamatus* exposed to heat stress. This agrees with the rise in the mRNA level of HIF-1α observed in the other organs (*i.e.* heart) of Antarctic *N. coriiceps* after acute exposure to elevated temperature (Beers and Sidell, 2011). This contrast with the observations in *C. aceratus* in which the cardiac level of HIF-1α remains unchanged in relation to increases of both temperature and $O_2$ (Devor et al., 2016). In the present study, we found that HIF-1α was localized in branchial mucocytes. In mammals, under pathological conditions, HIF-1α; was identified in globet cells of the large airways in which it is responsible of mucous production and globet cells hyperplasia (Polosukhin et al., 2011). The role of this hypoxia sensitive molecule in the branchial mucocytes of Antarctic species is unexplored. The possibility that in the gills of Antarctic teleost it may affect mucous production cannot be excluded. If this is the case, a modulation of the mucous barrier, together with the epithelial hyperplasia and oedema, and with the conceivable increase of the diffusional distances, may indicate, particularly in *C. hamatus*, that hypoxia really occurs as an effect of thermal stress. As proposed in humans, HIF-1α activation during acclimation to heat or hypoxia is accompanied by a parallel increase of HSPs system and this contributes to the systemic acclimation responses (Ely et al., 2014).

Under normal conditions, HSPs act as molecular chaperones that protect proteins from denaturation, assist in folding of the nascent protein and removes damaged proteins (Pelham, 1986; Geething and Sambrook, 1992). In fish, exposure to stress conditions, such as high temperature, induces a high expression of HSPs enabling the organisms to resist the insults of adverse stressors (Pelham, 1986; Geething and Sambrook, 1992; Petricorena and Somero, 2000 and references therein). In the gills of *C. hamatus* we demonstrated a constitutive expression of both HSP-70 and HSP-90 at the epithelial level of the secondary lamellae. While no data are available on the presence of HSP-90 in Antarctic teleost species, the presence of HSP-70 has been documented (Hofmann et al., 2000) under unstressed conditions. As expected, on the basis of the thermal dependent induction of the HSP system, in *C. hamatus*, we observed that after heat stress, HSP-90 and HSP-70 expression was stronger than under control conditions. In contrast, in *T. bernacchii*, HSP-90 was not detected while HSP-70 was expressed at the epithelial level only under control conditions. This is in agreement with the constitutive expression of HSP-70 reported in the gills of *T. bernacchii* by western blotting (Hofmann et al., 2000). Of note, after acclimation to high temperature, we found that the branchial HSP-70 expression disappears, suggesting that, in these teleosts, the gills do not possess an inducible heat shock response, as already proposed in *T. bernacchii* by Hofmann et al. (2000). It has been proposed that, in *T. bernacchii* the absence of heat stress response may correlate with the low levels of chemical and physical stressors found in Antarctic waters (Hofmann et al., 2000) and may contribute to the intolerance of this fish to increased temperatures (Robinson, 2008). It remains to be analyzed why, despite the same environmental

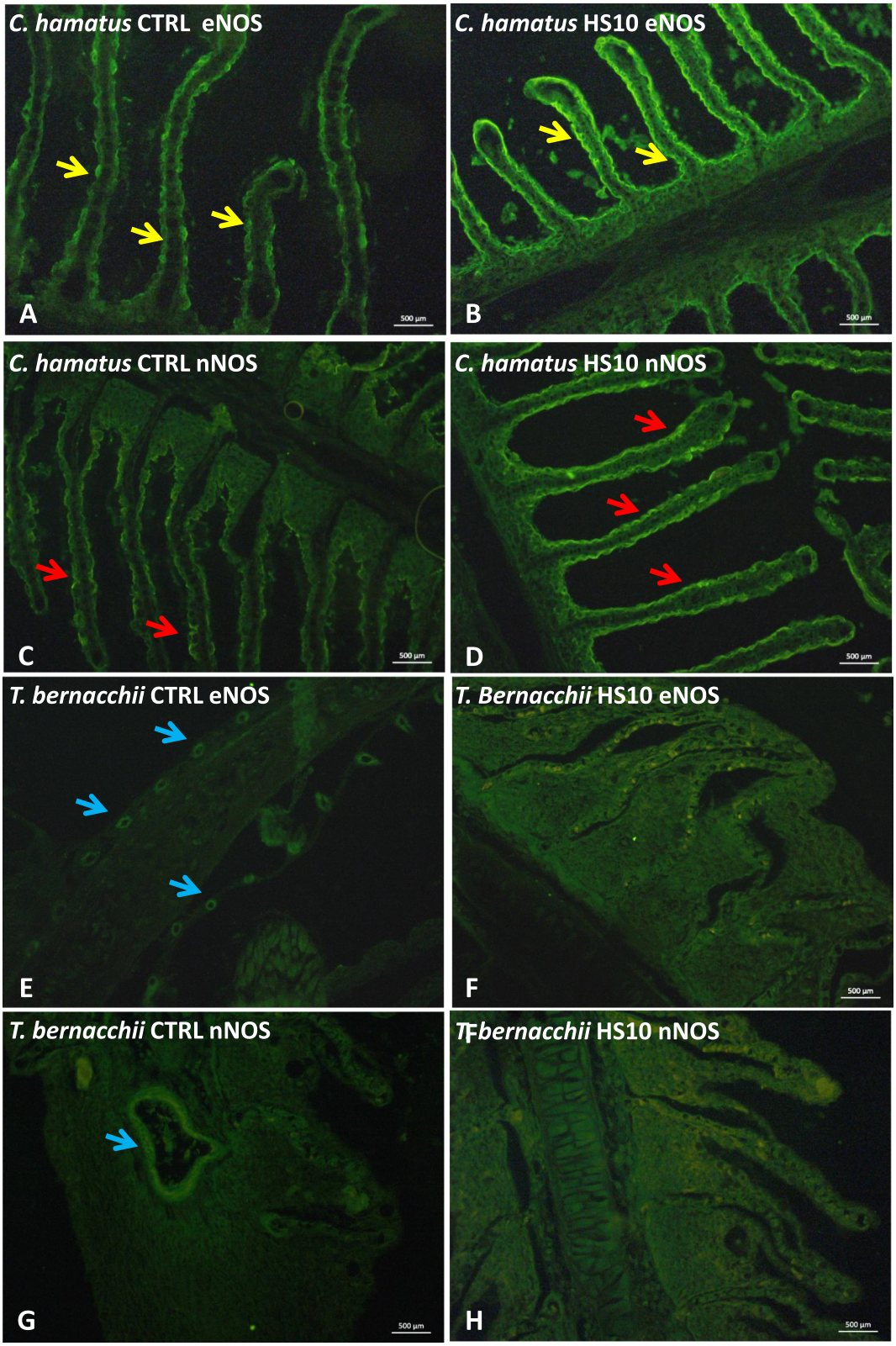


**Fig. 4.** eNOS (A,B,E,F) and nNOS (C,D,G,H) immunolocalization in the gills of CTRL (A,C,E,G) and HS10 (B,D,F,H) *C. hamatus* (A–D) and *T. bernacchii* (E–H). In *C. hamatus* both enzymes localize at level of respiratory epithelium. eNOS (yellow arrows); nNOS (red arrows). In *T. bernacchii* the eNOS and nNOS signal localizes at endothelial level (blue arrows) only in CTRL (E,G). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

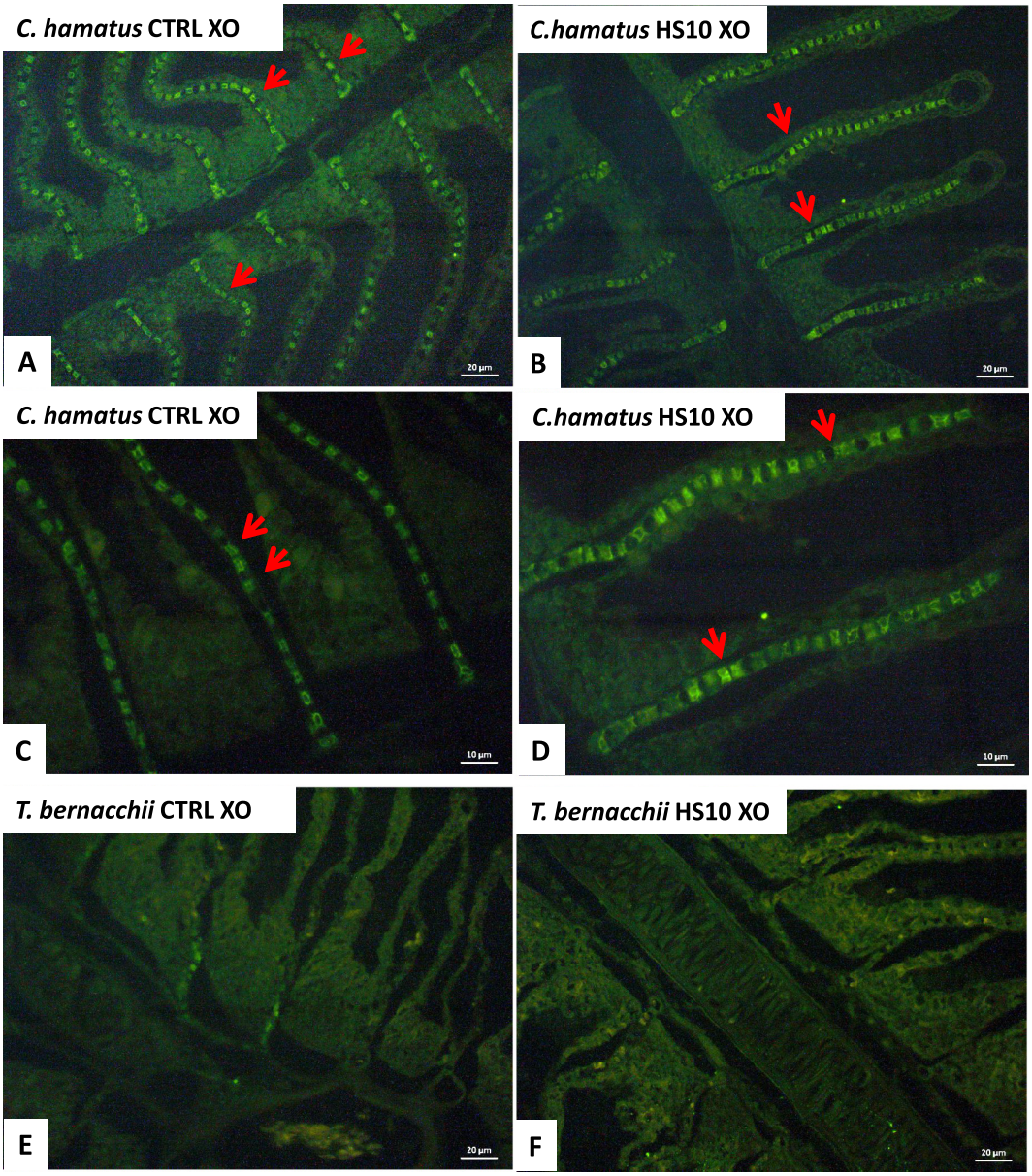


**Fig. 5.** Xantine Oxidase (XO) immunolocalization in the gills of *C. hamatus* (A–D) and *T. bernacchii* (E,F) in CTRL (A,C,E) and HS10 conditions (B,D,F). In *C. hamatus* the signal is confined at the level of pillar cells (red arrows). No signal was detected in *T. bernacchii*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

conditions, contrarily to *T. bernacchii*, *C. hamatus* maintains a heat shock response that in the gills may be represented by the activation of the HSPs system.

Of note, as observed in the gills of *N. rossii*, the hypoxia induced by heat stress determines a remarkable oxidative damage (Forgati et al., 2017). In several teleost species this is accompanied by an increase of enzymatic antioxidant activity (Forgati et al., 2017; Tolomeo et al., 2016). It has been suggested that, in these teleosts, an imbalance between the generation of ROS and the activity of the antioxidant systems alters the cellular redox state, thus generating oxidative stress (Forgati et al., 2017).

A major component of the oxidative response is the NOS/NO system (Forgati et al., 2017). In Antarctic fish, tissue hypoxia can induce NOS activation, leading to the formation of NO and RNS, together with the activation of antioxidant systems (Forgati et al., 2017 and references therein).

In *C. hamatus*, we observed by immunofluorence that, under control conditions both eNOS and nNOS are expressed in the gills at the level of the epithelium of the secondary lamellae. This expression increased after heat stress. These data that revealed the presence of the NOS/NO system also in the gills of these Antarctic species, already documented at the cardiac level (Garofalo et al., 2009b; Amelio et al., 2006), are indicative of a thermal susceptibility of this enzymatic system. It is possible that, as observed in temperate teleosts, such as the rainbow trout (McNeill and Perry, 2006) and the Atlantic croaker (Rahman and Thomas, 2015), the NOSs increment in *C. hamatus* could be related to the heat-induced hypoxia. On the contrary, in *T. bernacchii*, only a very low signal of both NOS enzymes was detected under control conditions, and disappears after heat stress. Recently, in the gills of catfish *Chaca chaca*, a similar localization pattern was detected for both eNOS and nNOS, whose expression is affected by hypoxia (Mistri et al., 2018). As previously suggested in other species (Garofalo et al., 2015a, 2015b and references therein), also in *C. hamatus* and *T. bernacchii* NO generated under CTRL conditions could contribute to the regulation of vascular tone, mucous secretion and osmoregulation. An interesting possibility, that deserves further investigations, is that the different localization of

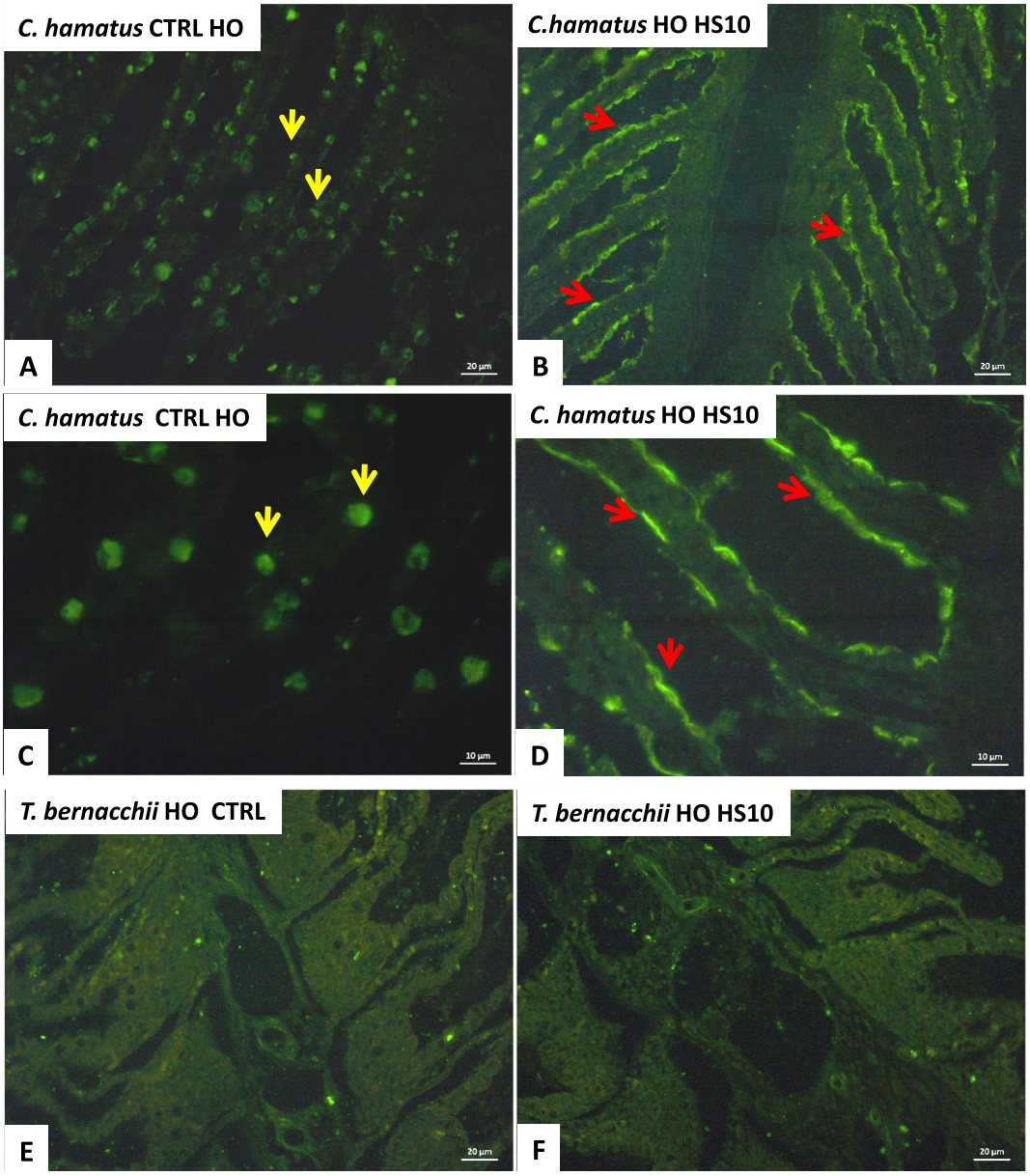


**Fig. 6.** Heme oxigenase (HO) immunolocalization of in the gills of *C. hamatus* (A–D) and *T. bernacchii* (E,F) in CTRL (A,C,E) and HS10 conditions (B,D,F). In CTRL *C. hamatus* the signal is confined in mucocytes (yellow arrows). In HS10, it appeared stronger and localized also at the respiratory epithelium (red arrows). No signal was detected in *T. bernacchii*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the enzymes between the two Antarctic species (epithelial: *C. hamatus*; endothelial: *T. bernacchii*), and the different response to heat stress, is associated with the absence/presence of respiratory pigments.

It is known that cells can generate NO from nitrite due to the nitrite reductase activity of XO (Baldissera et al., 2018). We previously documented that XO is present in *C. hamatus* and *T. bernacchii*, suggesting a role for this enzyme as source of NO, involved in the nitrite-dependent stimulation of myocardial contractility (Garofalo et al., 2015a, 2015b). In the gills of *C. hamatus* under both control conditions and heat stress, we observed that XO localized in the small vessels of the secondary lamellae. However, after heat stress, the signal was stronger than that detected under control conditions. This up-regulation of XO in heat stressed gills of *C. hamatus* may be considered an important pathway for ROS production, which could contribute to the branchial changes documented by our morphological analyses. It has been demonstrated, in the gills of the catfish, that XO contributes to inflammatory processes by activation of a pathway involved in ROS and NO production (Baldissera et al., 2018). The consequent oxidative damage could be counterbalanced by the parallel increase in the expression of antioxidant enzymes, such as HO. HO-1 is a heat-shock protein (Shibahara et al., 1987; Mitani et al., 1989) and a stress sensor induced by agents that cause oxidative damage (Keyse and Tyrrell, 1989; Nascimento et al., 1993). Under control conditions, in *C. hamatus* we demonstrated the presence of HO at the epithelial level of both primary and secondary lamellae. After heat stress, the signal appears stronger with respect to control animals. As reported in mammals, (Zhang et al., 2014; Foresti et al., 1997; Reiter and Demple, 2005; Kaizaki et al., 2006) HO acts as a cytoprotective enzyme in the epithelium and in other cell types. In addition, as shown in the respiratory epithelium of mammals and zebrafish, HO-1 may enhance the antioxidant potential, offering protection against injury induced by pollutants (Li et al., 2015). Based on these protecting properties, it can be suggested that, also in the respiratory epithelium of *C. hamatus*, HO can exert its antioxidant action. This is opposite to the situation encountered in *T. bernacchii*, in

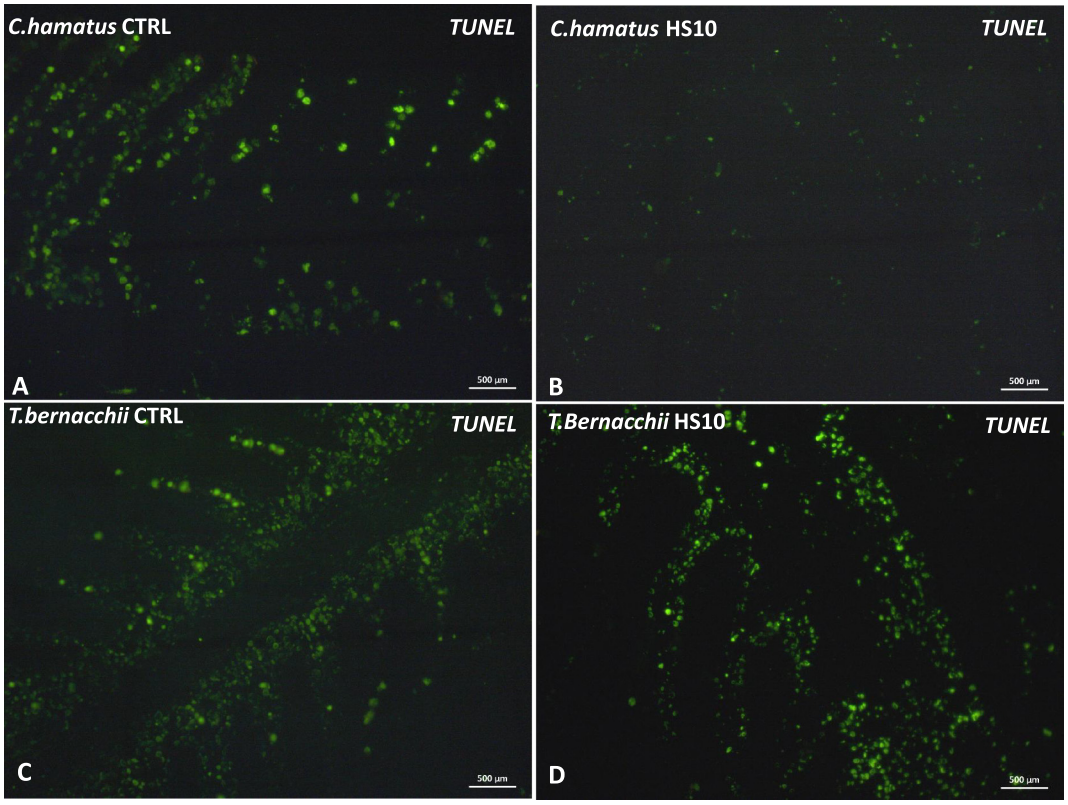


**Fig. 7.** TUNEL analysis of the gills of *C. hamatus* (A,B) and *T. bernacchii* (C,D) in CTRL (A,C) and HS10 (B,D) conditions. The apoptotic nuclei (green) decrease in HS10 *C. hamatus* whereas they notably increase in HS10 *T. bernacchii*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

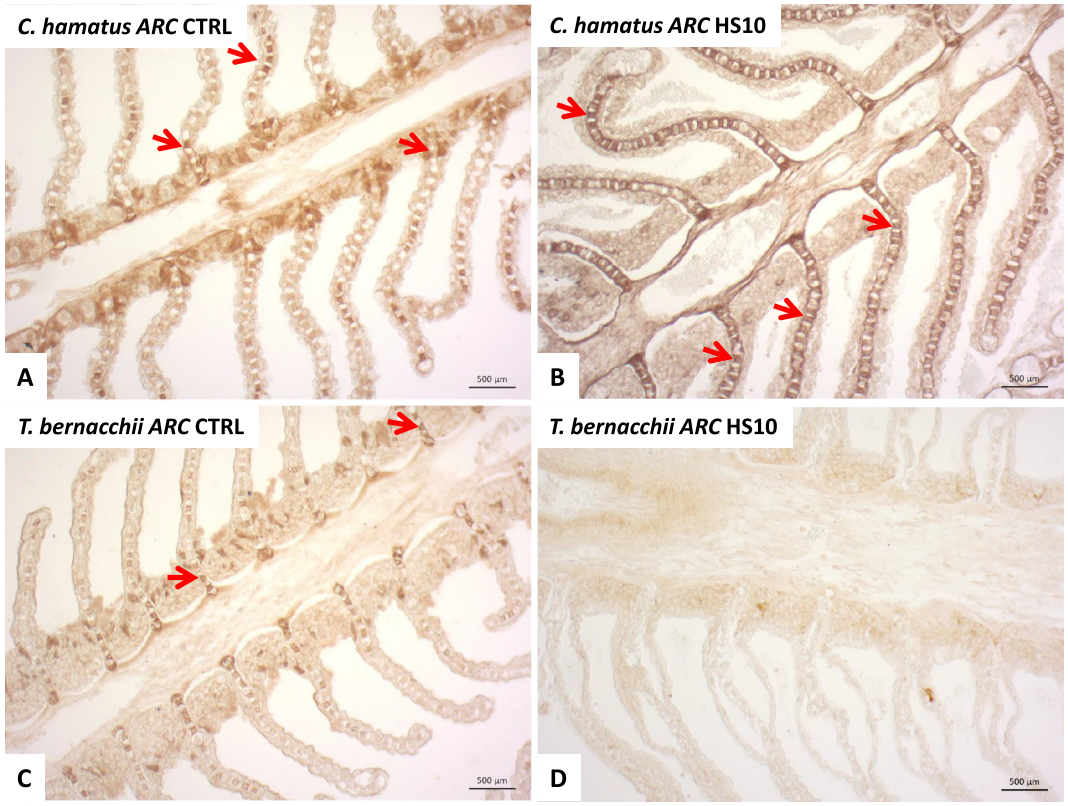


**Fig. 8.** Immunohistochemical localization of apoptotic repressor with caspase domain (ARC) in the gills of *C. hamatus* (A,B) and *T. bernacchii* (C,D). In CTRL of *C. hamatus* (A) and *T. bernacchii* (C) a weak signal is confined at the pillar cells (red arrows). After heat stress in *C. hamatus* (B) it is stronger and uniformly distributed whereas it disappears in *T. bernacchii* (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

which both XO and HO are not expressed, neither under control conditions nor after thermal stress. In *T. bernacchii*, exposed to 4 °C, Enzor and Place (2014) showed an oxidative damage up to 7 days, suggestive of a high compensation rate. The cell response to stress persists for 7 days, before returning to near basal level (Enzor and Place, 2014). It is possible that the absence of XO observed in *T. bernacchii* after heat stress (10 days), could contribute to re-establish basal conditions, possibly also reducing oxidative damage.

Many studies have reported that the antioxidant systems of Antarctic fishes are more efficient than those of tropical and temperate fish (Abele and Puntarulo, 2004; Grim et al., 2013). In fact, Antarctic fishes are less susceptible to oxidative damage despite the increased generation of ROS (Abele and Puntarulo, 2004; Grim et al., 2013). Therefore, as documented in other Antarctic fishes (*N. rossi* and *N. coriceps*), also in the species analyzed in the present study, a difference in the efficiency of the antioxidant systems can result in different level of oxidative damage in response to the stress (Forgati et al., 2017).

In parallel with the reduction of HSPs observed after heat stress in *T. bernacchii*, we detected a decrease of the anti-apoptotic protein ARC. Consistent with these data, by TUNEL technique, we observed an increase of apoptotic nuclei in the gills of *T. bernacchii* exposed to high temperature. Although cells have diverse protective mechanisms against stress, an enhancement of the stress beyond the ability of cells to cope with it may lead to cell damage, such as an increase of cell apoptosis (Chandra et al., 2000). On the contrary, in *C. hamatus*, we observed a reduction of apoptosis after heat stress, associated with an increment of ARC and HO. This is of relevance since, also in the gills of *C. hamatus*, as already documented in the respiratory epithelium of zebrafish (Li et al., 2015), HO may contribute to counteract apoptosis.

Our preliminary results suggest that, in both *C. hamatus* and *T. bernacchii*, heat stress could promote modifications of the branchial morphology. This is associated with modifications of molecules involved in the heat shock response and in the antioxidant defense. We found that, although the two Antarctic species experience the same environmental conditions, they exhibit a different response to heat stress. In particular, after heat stress, *C. hamatus* activates molecular protective mechanisms that are reduced or absent in *T. bernacchii*. Our data are in contrast with previous work, carried out on the heart, showing a major heat stress vulnerability of the icefish with respect to red blood species (Beers and Sidell, 2011).

A high plasticity and resistance of *T. bernacchii* to thermal stress has been documented (Podrabsky and Somero, 2006; Bilyk and DeVries, 2011). Recently, it was found in *T. bernacchii* gills and muscle that thermal stress affects lipids and fatty acids composition. These effects were suggested to contribute to maintain branchial and muscle function. Authors concluded that thermal stress may be associated with an acclimation response, rather than with pathological changes (Truzzi et al., 2018a, 2018b). In this view, also our preliminary results could be interpreted as a gills acclimation response to thermal stress. It remains to be established if the differences between the two species could be related to the presence or absence of the respiratory pigments. Very recently, O'Brien et al. (2018) demonstrate that in Antarctic fishes the level of oxidative damage and antioxidant molecules is organ dependent and cannot be related to the expression of Hb and Mb.

Acclimation to high temperature in Antarctic species induced a decrease in serum osmolality, which resulted from the positive compensation of $Na^+/K^+$-ATPase in osmoregulatory tissues (gills, kidney), without influence the activity of the pump in other organs (Gonzalez-Cabrera et al., 1995). Also in the present work, the modulation of apoptotic and oxidative stress markers in the gills of *T. bernacchii* and *C. hamatus* exposed to high temperature, could be organ specific without influences on animal survival.

Although more work is needed to fully depict the response of the gills of Antarctic teleost, our preliminary data may be of interest in the context of the predicted ocean-warming scenario in which, the increase of water temperature may severely affect the physiology of Antarctic stenotherm fish and thus their adaptative mechanisms.

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